PROTEOLYTIC ACTIVITY AT NEUTRAL pH IN BOVINE SPLEEN

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Proteolytic activity at neutral pH can be demonstrated in extracts from beef spleen. This activity is completely due to an aminopeptidase – or a number of aminopeptidases – which is able to hydrolyse proteins completely to amino acids. No evidence was found for the presence of endopeptidases active at neutral pH. The enzyme resembles to some extent swine kidney aminopeptidases.

Intracellular animal proteases active at acid pH have been extensively studied (for a review see refs. [1,2]), and these studies have led to the identification, separation and at least partial characterization of a number of enzymes involved. Proteolytic activity at neutral pH in animal tissue extracts, on the other hand, has often been mentioned, but reports on the isolation, separation or characterization of the enzymes are scarce ([3-8], and others). This is partly due to the much higher activities at acid pH values, but also to the rather elusive nature of the enzyme(s) assumed to be active at neutral pH. We directed our studies to the proteolytic activity possibly present in beef spleen, and especially to the problem of the existence of an endopeptidase active at neutral or slightly alkaline pH values. For the detection of this activity the traditional Kunitz method [9] using the absorbancy increase at 280 nm with casein as substrate, is hazardous [10]. We, therefore, used more sensitive and specific methods, viz. the Folin-reagent [11,12] and the ninhydrin method [13].

By both methods we were able to demonstrate proteolytic activity at neutral pH in spleen extracts. A comparison of the actual values obtained with both methods suggested that exopeptidase activity is predominant. Addition of proteins to the extracts did not increase the activity indicating that the enzyme(s) is (are) still saturated with endogenous substrate(s).

The activity could be optimally extracted in the following way: ground spleen is homogenized at pH 5, and the proteins soluble at this pH are extracted. The proteolytic activity is then extracted at pH 8 from the pH 5 sediment. Dialysis leads to a preparation which withstands freezing and can be stored at least for several months at -20°. This activity still containing the endogenous substrate has a pH optimum at 7.6 at 55° (optimum temperature; 60 min incubation) and 38° (fig. 1). The reaction products appeared to be mainly amino acids (Lys, Ala, Val, Leu, Gly, Thr, Arg, Ser, Glu, Phe, Tyr, Pro and Asp), but the presence of minor amounts of peptides could not be excluded.

We tried several different separation methods in order to obtain a substrate-free enzyme preparation, but without success. Gel-filtration chromatography on agarose (Sepharose 4B, Pharmacia, Uppsala, Sweden) showed that the enzyme and substrate migrate together in a high-molecular fraction with a molecular weight of one million or more as calculated from the elution of several hemocyanins. Probably, complexing of the enzyme(s) with other components, including its substrate, occurs.

A clear-cut separation of enzyme and substrate could, however, be achieved by exhaustive digestion of the substrate at 45° in a dialysis tube by which method inhibitory products appear to be removed. The enzyme obtained in this way is only active when substrates are added and the original activity can be nearly completely restored by adding boiled

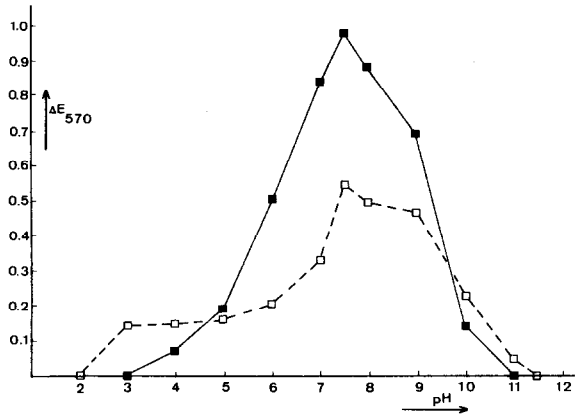

Fig. 1: *Autolysis as a function of the pH.* The increase in ninhydrin positive material (ΔE_{570}) after 60 min incubation is plotted against the pH of the incubation mixture. ■ at 55°; □ at 38°. The enzyme extract as described in the Experimental Part was diluted with an equal volume of Veronal buffer and the pH adjusted by addition of hydrochloric acid.

Table 1

Action on amino acid amides and peptides. For details see Experimental Part. Used abbreviations: Ac = acetyl; Be = benzyl; Bz = benzoyl; Cbz = carbobenzoxy; Et = ethyl; Me = methyl; NA = nitroanilide; Tosyl = toluenesulfonyl. The symbols ‖, |, ⁞, indicate rapid, medium and slow splitting, respectively.

AMINO ACID AMIDES		DIPEPTIDES		TRI-, TETRA-, PENTAPEPTIDES	
SPLIT:	NOT SPLIT:	SPLIT:	NOT SPLIT:	SPLIT:	NOT SPLIT:
Ala ‖ amide		Gln ⁞ Asn		Gly + Gly – Gly	
	Asp-diamide	Glu ⁞ Phe	Cbz-Glu-Phe	Gly ‖ Leu + Gly	
	Gly-amide		Cbz-Glu-Tyr	Gly ‖ Phe – Glu	
	His-amide		Cbz-Gly-Glu-OBe	Gly ‖ Phe ‖ Glu – diamide	
Leu ‖ amide			Gly-Gly	Gly ‖ Phe + Gly	
Leu ‖ β naphtylamide			Gly-Gly-OMe	Gly ‖ Phe ‖ Phe	
Met ‖ amide		Gly ⁞ Leu	Gly-D-Leu	Gly ‖ Phe – D-Phe	
Phe + amide		Gly + Phe	Gly-D-Phe	Gly ‖ Phe ‖ Phe – amide	
	Ser-amide		Cbz-Gly-Phe	Leu ‖ Gly – Gly	
	D-Ser-amide	Gly ‖ Phe ‖ amide		Pro ‖ Tyr ⁞ Gly	Cbz–Pro–Tyr–Gly
Trp + amide		Gly ‖ Phe ‖ (N-Me)-amide		Pro ‖ Tyr ‖ Gly- amide	
	N-Ac-Tyr-amide	Gly ‖ Phe + pNA		Val ‖ Tyr – Pro	
	N-Ac-Tyr-OEt	Gly + Trp			
Val ⁞ amide		Gly + Tyr		Gly ‖ Phe ‖ Gly – D-Phe	
	Bz-Arg-amide	Gly ‖ Tyr ⁞ amide		Pro ‖ Phe ‖ Gly ⁞ Lys	
	Bz-Arg-OEt	His + Lys		Pro ‖ Tyr ‖ Gly – Asp-(β) OMe	
	Bz-Arg-pNA	Leu ⁞ Glu			Cbz–Pro–Tyr–Gly–Gly–OMe
	Bz-Phe-OMe	Leu ‖ Glu – diamide		Pro ‖ Tyr ‖ Gly – Pro	
		Leu ⁞ Gly	D-Leu-Gly	Pro ‖ Tyr ‖ Gly + Tyr	
		Leu ‖ Met + amide			
		Leu + Phe	Leu-D-Phe	Val ‖ Lys ‖ Val ‖ Tyr – Pro	
		Leu + Trp			
		Leu + Tyr			
			N_{α}-Tosyl-Lys-Gly-OEt		
		Lys ‖ Lys			
		Phe ‖ Arg			
			Pro – Phe		
		Pro + Tyr	Cbz-Pro-Tyr		
		Pro ‖ Tyr ⁞ amide			
			Cbz-Ser-Gly-amide		
			Val-Gly		

extract which apparently contains saturating amounts of substrate.

The substrate-free enzyme preparation was now used to study the specificity and several other properties of the enzyme. The enzyme is, as will be fully reported later, stable for one hour between pH 4 and 11 at 4° and is inhibited by several metal ions (Ni^{++}, Zn^{++}, Cd^{++}, Hg^{++}, Pb^{++} and Co^{++}). Mg^{++}, Mn^{++} and EDTA have no effect on the enzyme; alcohols inhibit, the inhibition increasing with increasing chain length.

Table 1 shows the action of the enzyme on a number of amino acid amides, peptides and peptide derivatives. It is clear that the enzyme is an aminopeptidase with a rather broad specificity, and absolutely requires that both amino acids forming the hydrolysable bond should have the L-configuration. Polar side chains (Asp-diamide, Ser-amide) or a free carbo-

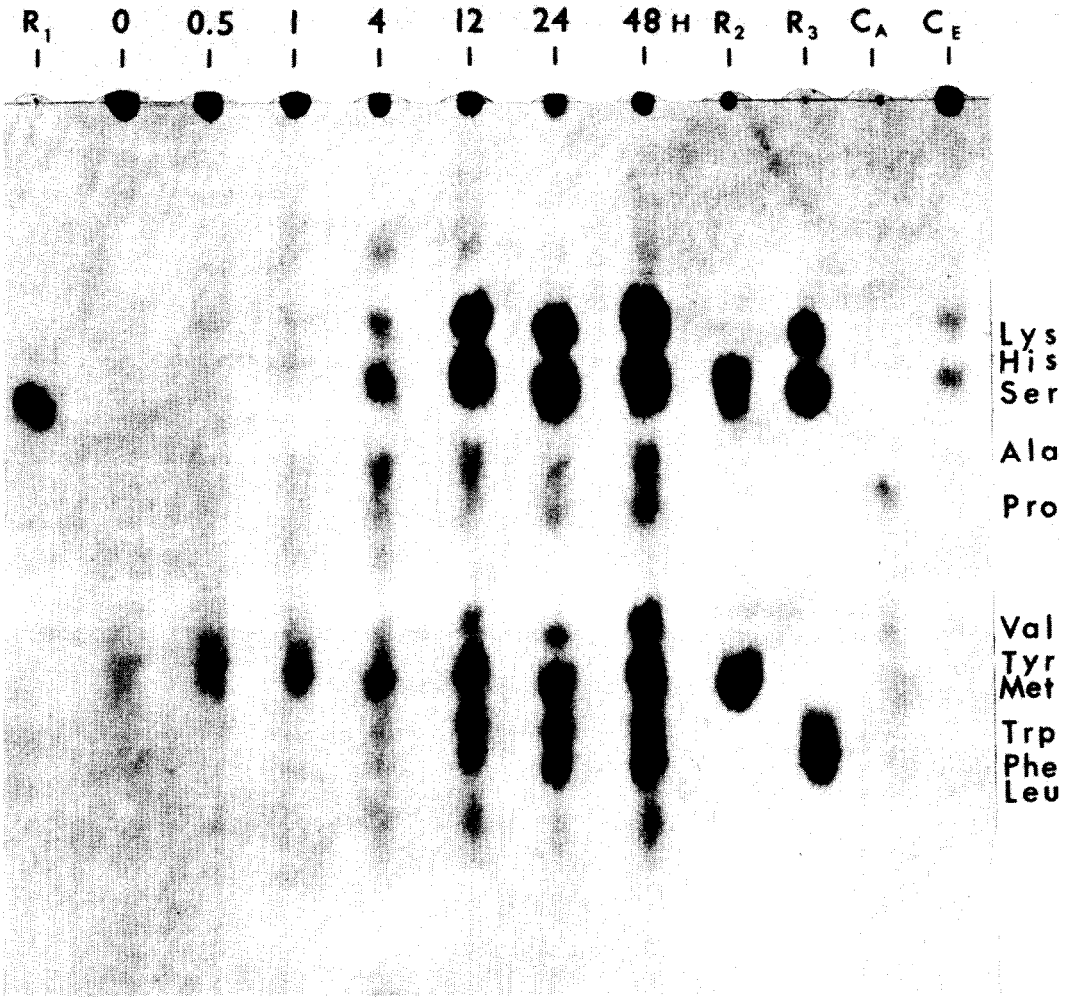

Fig. 2: *Time course of ACTH hydrolysis.* 2.5 mg ACTH (kindly provided by N.V. Organon, Oss, The Netherlands) were dissolved in 0.25 ml Veronal buffer pH 7.6 and mixed with 0.25 ml enzyme preparation. At the times indicated, 50 µl samples were subjected to paper chromatography. For the enzyme preparation and chromatography see Experimental Part. R_1, R_2, R_3 are reference amino acids or amino acid mixtures; C_A = no enzyme added; C_E = no ACTH added.

xyl group on the second amino acid (Gly-Phe-amide versus Gly-Phe, Gly-Tyr-amide versus Gly-Tyr, Leu-Glu-diamide versus Leu-Glu) decrease the rate of hydrolysis considerably. In these respects the enzyme resembles pig kidney leucinaminopeptidase [14].

In other respects, it is however different. Neither Mn^{++} nor Mg^{++} have any effect while the pH activity curve is rather different and resembles more that of another aminopeptidase from swine kidney [15]. The existence of a leucinamide splitting peptidase in spleen has already been shown by Fruton et al. [16].

Polylysine is attacked by our preparation. Chromatographic analysis of the products according to Waley and Watson [17] showed lysine as the only product, thus excluding the presence of an endopeptidase in our preparation.

The enzyme was active against all proteins tested, whether native or denaturated (hemoglobin, bovine serum albumin, casein, oxidized ribonuclease, insulin-B chain, glucagon). In order to decide whether it can lead to the complete sequential split of a protein, β corticotropin (ACTH) [18] was used as a substrate and the products studied at several time intervals (fig. 2) and after exhaustive splitting (fig. 3). It is obvious from these figures that this molecule is completely split to amino acids by our preparation.

Our results show clearly that spleen contains an enzyme, or a number of enzymes, able to split pro-

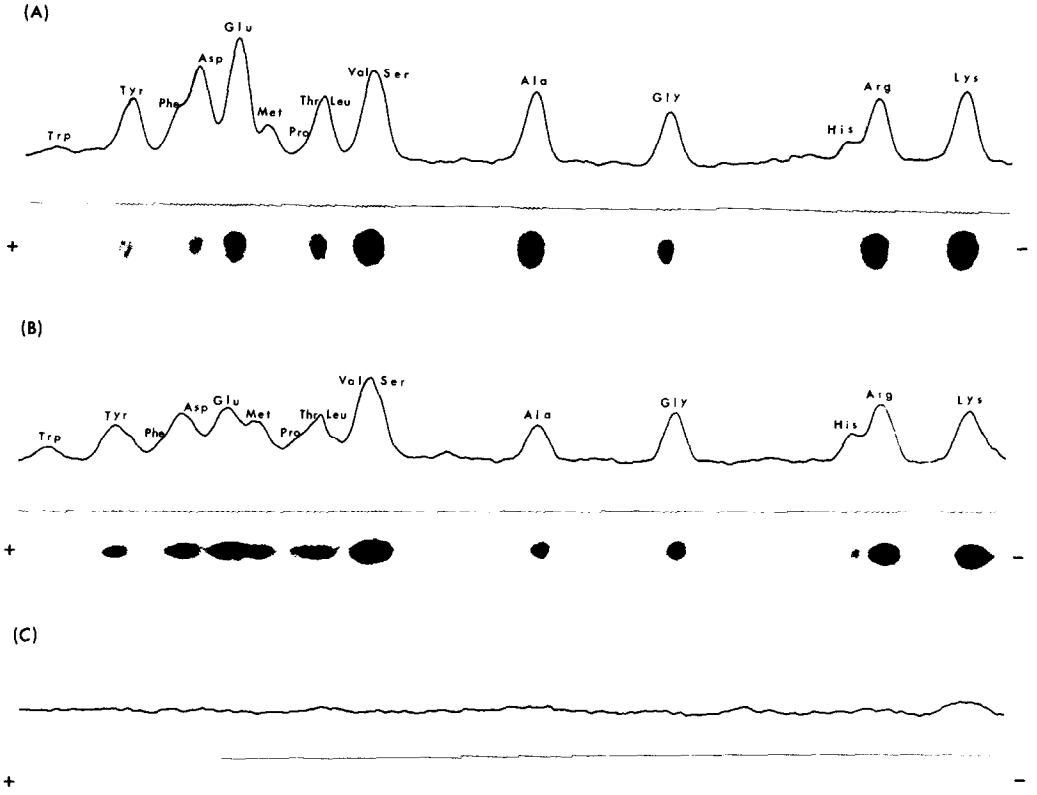

Fig. 3. *Analysis of ACTH degradation products.* ACTH and the enzyme preparation were incubated for 48 hours as described in the legend to fig. 2. 10 µl of the reaction mixture at 0 h (C) and 48 h (B) were subjected to paper electrophoresis: Gilson Medical Electronics electrophorator; 6% formic acid pH 1.86, 3 h, 4000 V, 125 – 150 mA, paper dimensions 25 – 130 cm. The spots were stained according to [19] and scanned by means of a Beckman RB Analytrol at 550 nm. For comparison, an acid hydrolysate of ACTH (6 N HCl, 72 h; 111°) was used (A).

teins to amino acids unspecifically and completely from the N-terminal. This makes the assumption of endopeptidases active at neutral pH, for which we obtained no evidence whatsoever, unnecessary. Intracellular protein breakdown at neutral pH proceeds – at least in beef spleen, but probably in other animal tissues as well – in a different way from the extracellular protein breakdown in the digestive tract, and the intracellular proteolysis at acid pH values.

Experimental part

Veronal buffer (10 mM): 400 ml of 0.025 M diethylbarbituric acid and 14.5 ml of 0.5 M sodium diethylbarbiturate were diluted with water to 1725 ml. Where necessary the pH is adjusted with dilute HCl.

The *enzyme extract* is obtained by grinding 1 part of spleen in 2 parts Veronal buffer pH 5 in a Potter Elvehjem homogenisator. After standing for one hour at 2° the homogenate is centrifugated (MSE 18 centrifuge, angle rotor nr 69179; 12000 rev./min; 23000 × *g*; 60 min; 2°). The sediment is stirred for 30 min with the original volume of Veronal buffer pH 8. The clear red supernatant, obtained after centrifugation, is dialysed against this buffer.

The *enzyme preparation* is obtained by incubating the extract at 45° in dialysis tubing for 32 h.

Activity Assay (modification of [6]): In autolysis experiments 0.4 ml enzyme *extract* is diluted with 0.4 ml Veronal buffer of the desired pH (7.6 except for the experiment in fig. 1). When the enzyme *preparation* was used the substrates were dissolved in the buffer. After 0 and 60 min incubation at 55°, 4 ml ice-cold 0.2 M sodium acetate buffer pH 5.2 were added. The mixture was placed in a boiling-water bath for 4 min. After standing for 30 min the precipitate was filtered off through Whatman 3 MM paper. In the filtrate ninhydrin-positive material was determined according to Moore and Stein [13].

Action on synthetic substrates: Solutions of the synthetic substrates (final concentrations 5 mM) (table 1) were incubated in Veronal buffer pH 7.6 with once-diluted enzyme preparation at 37°. At t=0 and t=60 min – for some substrates also at t=15, 30 and 45 min – 25 µl samples were transferred to the chromatography paper, and the reaction stopped by drying in hot air.

Descending *chromatography* was carried out on Schleicher and Schüll chromatography paper (nr 2043 b MgL.) with butanol/acetic acid/pyridin/water (15:3:10:12 v/v) during 16 h after 6 h equilibration. After drying the spots were stained with ninhydrin-copper reagent [20], isatin reagent [21] or according to Reindel and Hoppe [22].

Peptides: A large part of the peptides and similar components mentioned in table 1 was available from commercial sources (Fluka, Hoffmann-La Roche, Mann Research Lab., Yeda); others were prepared by Miss Jeanette Gorter in our laboratory to whom we are greatly indebted.

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